

Metagenomic assessment of the potential microbial nitrogen pathways in the rhizosphere of a Mediterranean forest after a wildfire

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Abstract

Wildfires are frequent in the forests of the Mediterranean Basin and have greatly influenced this ecosystem. Changes to the physical and chemical properties of the soil, due to fire and post-fire conditions result in alterations of both the bacterial communities and the nitrogen cycle. We explored the effects of a holm-oak forest wildfire on the rhizospheric bacterial communities involved in the nitrogen cycle. Metagenomic data of the genes involved in the nitrogen cycle showed that both the undisturbed and burned rhizospheres had a conservative nitrogen cycle with a larger number of sequences related to the nitrogen incorporation pathways and a lower number for nitrogen output. However, the burned rhizosphere showed a statistically significant increase in the number of sequences for nitrogen incorporation (allantoin utilization and nitrogen fixation) and a significantly lower number of sequences for denitrification and dissimilatory nitrite reductase subsystems, possibly in order to compensate for nitrogen loss from the soil after burning. The genetic potential for nitrogen incorporation into the ecosystem was assessed through the diversity of the nitrogenase reductase enzyme, which is encoded by the *nifH* gene. We found that *nifH* gene diversity and richness were lower in burned than in undisturbed rhizospheric soils. The structure of the bacterial communities involved in the nitrogen cycle showed a statistically significant increase of Actinobacteria and Firmicutes phyla after the wildfire. Both approaches showed the important role of Gram-positive bacteria in the ecosystem after a wildfire.

Keywords:

Metagenomics; microbial communities; nitrogen cycle; rhizosphere; wildfire; Mediterranean forest

Introduction

As occasional natural events, wildfires probably influenced the vegetation of the Mediterranean Basin before the arrival of humans [1]. Their effect, in combination with regular drought, high temperatures and grazing over thousands of years, shaped the development of a specific type of adapted vegetation [2]. This is the case for the holm oak (*Quercus ilex* subsp. *ballota*), which displays regrowth after fires. Holm oak formations cover a total area of 9.66×10^6 ha in Spain, but they rarely form entire forests, due to their management as a pastoral woodland called *dehesa* [3], and the effects of fire and clear-cutting. The area affected by wildfires in Spain reached an annual mean of 127,209 ha in the period 2000-09, and the affected landscapes included shrubs, forests, and herbaceous formations [4]. These fires included a wildfire in the Sierra Nevada National Park in South East Spain in September 2005, which affected 3416.74 ha [5], including 412 ha dominated by holm oaks.

The high temperatures reached during the wildfire cause immediate changes in the physical and chemical properties of the soil, the magnitude of which depends on the fire severity and soil type [6, 7]. These changes, together with the environmental conditions prevailing after the fire, may cause alterations in the soil's biological characteristics, such as microbial biomass and activity [6, 8, 9]. They may also modify the taxonomic structure of the soil's microbial communities, with an increasing proportion of spore-forming bacteria [10-12]. Understanding the processes that contribute to the recovery of the soil and the microbial communities could help to re-establish the plant formations of the burnt area.

Available nitrogen (N) and water are the most common limiting factors in both natural and agricultural ecosystems [12, 13]. Remarkably, after wildfires, an ephemeral flush in mineral N can often be observed [6, 9]. In a recent meta-analysis, Wang et al. [9]

concluded that wildfires in Mediterranean forests typically have a positive effect on soil organic carbon (C) and N pools, but a negative effect on N mineralisation, increasing the likelihood of N losses from forest ecosystems due to leaching and decreases in microbial activity. The soil bacteria are essential components of the biogeochemical N cycle, but there is only little study on their presence in post-fire environments [14].

The biological incorporation of molecular nitrogen (N₂) in the ecosystem is only possible by means of its fixation by diazotrophic prokaryotes, a reaction carried out by the nitrogenase enzyme that is composed of two subunits, the MoFe protein and the Fe protein. The *nifH* gene encodes the Fe protein, which acts as the nitrogenase reductase, and the high level of conservation of this gene and its presence in all diazotrophic bacteria make it an ideal molecular marker [15]. The molecular analysis of this gene has been used to study nitrogen-fixing bacteria after a forest fire [12], where, despite the overall decrease in microbial biomass, including that of nitrogen-cycling bacteria, the nitrogen-fixing community actually became more diverse within a month of the fire.

However, other authors have concluded that the relative richness and evenness of these communities decreases 16 months after various types of fire [16]. Other genes playing a key role in the N cycle have been used as markers for different stages of this biogeochemical cycle. For example, the *nosZ* gene has been used to study denitrification, and the *amoA* gene has been used to study ammonia oxidation [17].

Nevertheless, to our knowledge, no study of the entire N cycle after a wildfire has been carried out before, despite the need to understand the reaction of the ecosystem in terms of the strength of N incorporation pathways after a catastrophic event. However, high-throughput shotgun sequencing has been applied to different environmental samples, in order to characterize the nitrogen metabolism [18-20].

We investigated the dynamics of microbial communities and their potential role in the N-cycle within the holm oak rhizosphere three years after a wildfire. Bacteria are early indicators of environmental changes, due to the important role they play in various biogeochemical cycles and their sensitivity to environmental changes. Therefore, we carried out a metagenomic analysis of nitrogen metabolism, following the direct pyrosequencing of total soil DNA extracted three years after the wildfire. Since we did not detect any structural genes of the nitrogenase enzyme with this technique, we used clone libraries to assess the diversity of the *nifH* gene, to investigate changes in potential nitrogen fixation during the recovery of the ecosystem.

Methods

Experimental sites

The study area is located in the Sierra Nevada Natural and National Park (SE Spain), where a wildfire in September 2005 burned 3426.74 ha, including 412 ha of evergreen holm oaks (*Quercus ilex* subsp. *ballota*). Soil samples were collected in the valley of the Lanjarón River where three sites were selected: two in areas directly affected by the wildfire (burned forest containing holm oak trees [BOF] and burned bulk soil [BBS] covered by grasses and shrubs) and a nearby site in evergreen, undisturbed oak forest (UOF, Fig. S1).

Three sampling plots were randomly chosen within each study site along transects of 1.0 km length. At the BOF and UOF sites, we sampled the rhizosphere of three trees per plot, each with a diameter of at least 15 cm at breast height, and separated by at least 5 m. In the BBS plots, we took an equivalent number of samples from bulk soil. For all sites and plots, sampling took place on April 29, 2008 (three years after the wildfire).

The BBS site was on a terraced slope, whereas the BOF and UOF sites were on a steep slope, all of them south-facing. The soil sampling points and the sampled trees were marked, and the positions of all sites were registered with the global positioning system (GPS, Fig. S1).

Sample collection and soil chemical analysis

The rhizospheric samples were collected by following the tree's main roots until young, cork-free roots were found at a distance of less than 50 cm from the trunk, where we took soil that was attached to the roots. The soils from the sampling areas were loams, with the exception of the one from the BBS site, which was sandy loam (Table 1). All these soils are classified as haplic phaeozems of siliceous origin. At each sample point, we collected soil at depths of 5 to 25 cm, which we stored immediately at 4° C until processing. The elimination of the first 5 cm of the soil allowed us to discard minor roots from herbaceous plants, which ensured the influence of the tree rhizosphere on the microbial communities. We sieved the soil samples through a 2 mm mesh. Then we processed 2 kg of soil from each site for physicochemical analysis, including soil type, pH, available water, salinity, total nitrogen, organic matter, assimilable phosphorous, magnesium and calcium. All physicochemical analyses were carried out using standardized procedures at the Food and Agriculture Laboratory of the Andalusian regional government at Atarfe (Granada, Spain) [21] which methodology is listed in the supplementary material.

DNA extraction and deep-sequencing

Within 24 hours after sample collection, we extracted DNA from each soil sample with the PowerSoil DNA Isolation kit (MoBio Laboratories Inc.), following the

manufacturer's recommendations. Aliquots of total-community DNA were then used for PCR-based analyses of diazotrophic communities. For each sampling site, DNA from the rhizosphere of nine trees of the three plots was pooled in equal amounts to obtain 5 µg. The DNA from BOF and UOF samples was subjected to pyrosequencing with the Roche 454 GS FLX Titanium platform at LifeSequencing SL (Valencia, Spain), on a quarter of a plate in order to obtain *c.* 100 Mb of information from each sample. The raw metagenomic reads were filtered for replicated sequences with the 454 Replicate Filter web tool [22], with a 0.9 sequence identity cutoff, a 0 length difference requirement and 10 starting base pairs to be checked. The SeqTrim pipeline with default parameters [23] was then used to filter out sequences of low complexity and quality. The trimmed metagenomic sequences for the UOF and BOF sites, with an average length of 387 and 393 bp, respectively, were assigned to SEED subsystems categories and KEGG orthology database on the MG-RAST web server [24], with a maximum *e*-value cut-off of e^{-10} , a minimum identity cut-off of 60% and a minimum alignment length of 50 bp. SEED subsystems and KEGG orthology are databases with a categorization system that classifies gene functional categories into a hierarchy with four levels of resolution, in which the finest level of resolution is the function or orthology protein group, respectively. The sequences from this study are publicly accessible from MG-RAST server under the codes 4465556.3 for UOF and 4465558.3 for BOF. The MG-RAST table format of sequences associated with nitrogen metabolism on SEED subsystems database was transformed to STAMP [25] format and the metabolic features of the two samples were statistically compared on STAMP software by two-tailed Fisher's exact tests with Newcombe-Wilson confidence intervals and Storey FDR correction, with a minimum *p*-value cut-off of 0.05. Metagenomic sequences associated with the nitrogen metabolism on the KEGG orthology database

(ko 00910) were phylogenetically classified by BlastX against the NCBI non-redundant protein database (nr), with a maximum e-value cut-off of e^{-10} , a minimum identity cut-off of 60% and a minimum alignment length of 50 bp, taking the closest affiliations with known genera on the basis of sequence similarity. Taxonomical abundances were compared, using the METASTAT web server [26].

nifH gene amplification, libraries construction and sequence analysis

Since no sequences of the *nifH* gene were obtained in the metagenomic analysis, we decided to explore the nitrogen fixation pathway by the construction of gene libraries. In this approach, the burned bulk soil (BBS) was used as control, since three years after the wildfire it was covered by the nitrogen-fixing leguminous shrub *Adenocarpus decorticans*. The *nifH* gene was amplified by PCR with the primers described by Widmer et al. [27], by a nested PCR as described by Villadas et al. [28] to obtain a 370 bp amplicon. Gene libraries were generated from an equimolecular mix of the PCR products for the three trees from each plot. In this way, we obtained a representative sample for the plot, thereby minimizing the potential bias associated with the use of a single sample from a single tree. The pooled PCR products were cleaned by centrifugation on Illustra MicroSpin™ S-300 HR columns (GE Healthcare) according to the manufacturer's instructions. These *nifH* fragments were then ligated into the pGEM-T Easy vector (Promega) and used to transform *E. coli* strain DH5 α , according to Villadas et al. [28]. Positive bacterial colonies were selected (at least 50 from each sampling site) on appropriate LB agar plates, and the sizes of the inserts were checked by PCR with T7 and SP6 primers. PCR products of the correct size were sequenced after cleaning by centrifugation on Illustra MicroSpin™ S-300 HR columns. The integrity, size and quantity of DNA was checked by gel electrophoresis, according to

standard procedures. Clones were sequenced by the Sanger method, with an ABI Prism 3130XL Genetic Analyzer, according to the manufacturer's instructions.

Raw sequence data was processed in Sequence Scanner version 1.0. Sequence alignments were generated on the MAFFT 6 server [29] and the distance matrix was obtained with the Phylip dnadist program. MOTHUR [30] software was used to determine the structure of microbial communities and to obtain the number of operational taxonomic units, OTUs, (S, observed richness) with a sequence similarity threshold of 93%, the Chao1 index (Chao1, expected richness) and the Shannon index (H' , diversity), according to default parameters of MOTHUR software. Pielou index (J' , evenness) was calculated using the formula $J' = H' / \ln(S)$ and coverage (G) was obtained according to the Good's coverage index ($G = 1 - n / N$; n = number of singletons, N = number of sequences).

BlastX comparison with the NCBI non-redundant protein database (nr) was performed for each of the *nifH* sequences for the taxonomic assignment, taking the closest affiliations with known diazotrophs based on the sequence similarity with a maximum cut-off of e^{-10} .

Comparative studies, analyzing differences between sequences of the various gene libraries were performed with J-LIBSHUFF [31], which can compare more than two libraries simultaneously. We used the statistical tool Ginkgo [32] to compare libraries by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering, with the Euclidean distance matrix of the relative abundance of each OTU in each sample.

All phylogenetic assignments and abundances of bacterial groups presented in the results are based on the similarity of the proteins involved in the nitrogen cycle.

The sequences of the partial *nifH* clone libraries were deposited in the NCBI database under accession numbers KC667152 to KC667559, with the exception of the BOF library whose accession numbers are KC551480 to KC551532.

Results

Physicochemical properties of the soils

One of the first impacts fire has on the ecosystem is the modification of the physical and chemical characteristics of the soil, leading to other long-term changes. Our study was conducted three years after the wildfire, therefore the intensity of the impact on the soils could have been modified by other environmental factors. However, even after three years, the soil was more alkaline at the BOF site (pH 7.6) than at the other sites, which were slightly acidic (pH 6.1 at UOF and pH 6.5 at BBS; Table 1). Moreover, the area of burned holm oaks (BOF) had the highest salinity (0.22 mS/cm), the highest contents of magnesium (7.2 mg/kg) and calcium (17.97%) and the lowest assimilable phosphorus concentration (5.2 mg/kg), whereas the other soil affected by the wildfire (BBS) had the lowest salinity (0.08 mS/cm) and the highest assimilable phosphorus concentration (20 mg/kg). Total nitrogen and organic matter contents were lower in the soils affected by the wildfire (BOF and BBS) than in the unburned soil (UOF), however, the C/N ratios were similar in the three soils (Table 1).

Metagenomic analysis of the nitrogen cycle

The DNA obtained from the rhizospheres of burned (BOF) and non-burned (UOF) holm oak in the spring of 2008, three years after the wildfire, was pyrosequenced. In total, 316973 reads were obtained from the BOF soil and 520430 from the UOF soil, yielding

257697 and 412302 sequences after trimming, respectively. The mean length of the trimmed sequences for the BOF soil was 393 nucleotides, yielding 80 Mb of information. For the UOF soil, the mean length of the trimmed sequences was 387 nucleotides, yielding 160 Mb of information. Using the MG-RAST web server, we obtained 115406 features assigned to identified functional categories for the BOF soil and 171602 for the UOF soil. A heatmap comparison of both metagenomes showed no statistically significant differences at the level of metabolic groups (data not shown). At the subsystem level 1 (functional groups), only 1007 of the BOF sequences and 1515 of the UOF sequences were related to proteins involved in N metabolism. An analysis of the various functions involved in this process, showed that at subsystem level 2 (N-metabolism), more than 52% of the sequences were related to ammonia assimilation (Fig. 1) at both sites, with sequences related to nitrate and nitrite ammonification the next most frequent (around 16%). The functions classified by MG-RAST in the SEED subsystem level 3 database included nitric oxide synthetase and nitrosative stress, which comprised around 9.1% and 0.65% of the total number of sequences per sample, respectively. Nitrogen fixation accounted for 20 sequences (1.33%) in the UOF and 23 sequences (2.36%) in the BOF. Remarkably, none of the identified N₂-fixation proteins displayed any similarity to NifH in either of the two rhizosphere types. Instead, all the identified sequences displayed similarity with regulators, such as those encoded by *nifA*, *vnfA* or *ntrC*. There were no statistically significant differences between sites in the afore-mentioned functions; in contrast, the proportion of sequences involved in denitrification differed significantly between sites, accounting for 2.33% of the sequences at the UOF and only 1.03% at the BOF site (Fig. 1). Dissimilatory nitrite reductase was represented in a higher proportion of sequences in the UOF (4.19%)

compared to the BOF soil (2.05%; $p < 0.01$), whereas allantoin utilization was better represented in the BOF (9.74%) compared to the UOF soil (5.18%; $p < 0.01$; Fig. 1). The phylogenetic assignment of the bacterial communities based on the similarity of the proteins involved in the nitrogen cycle showed that more than 80% of all proteins were related to bacteria of the phyla Bacteroidetes, Proteobacteria and Actinobacteria (Fig. 2A). When the phyla Acidobacteria, Firmicutes and Planctomycetes were included, the percentage of the sequences reached around 90%, with only 5% of proteins unassigned to any bacterial taxa. Of these phyla, only Actinobacteria and Firmicutes were more abundant in the BOF rhizosphere than in the UOF ($p < 0.05$) three years after the wildfire (Fig. 2A). The phyla Bacteroidetes and Proteobacteria were the more abundant in the unburned UOF site than in the burned site, although the difference between the UOF and BOF sites was not statistically significant. The analysis at the genus level, with a cut-off above 2% of the total sequences, showed that there were four genera, which significantly increased in abundance after the wildfire: *Arthrobacter*, *Bacillus*, *Blastococcus* and *Spirosoma* (Fig. 2B).

Analysis of diazotrophic communities

In total, 154 partial sequences (370 bp) of the *nifH* gene were obtained, with a minimum number of 50 sequences per sample. This number of sequenced clones was sufficient to obtain a representative assessment of *nifH* gene diversity based on rarefaction curves (Fig. S2) and a coverage of between 84% for the UOF soil and 94% for the BBS soil (Fig. S2). The diazotrophic community of BBS was more similar to that of UOF, whereas the presence of burned trees in the BOF resulted in a more differentiated diazotrophic community. Agglomerative hierarchical clustering analysis of the abundances of each OTU, showed that BBS and UOF communities were separated by a

distance of 46% while the BOF community was joined, to the BBS and UOF branch, at a distance of 62% (Fig. S3). J-LIBSHUFF analysis showed that all samples differed from one-another, although the diazotrophic community in the BOF soil could be regarded as a subsample of the UOF community (p-value = 0.0176).

The UOF soils had the highest number of OTUs, with a richness of 17 OTUs at 93% similarity, whereas richness was lowest for BOF (S=9) and intermediate for BBS (S=13, Fig. 3A). The value of the Chao1 richness estimator was higher for the UOF rhizosphere (26) than for the samples from the burned sites (14). The diversity, as measured by Shannon's index, was higher for the unburned than for the burned soils; but the diversity in the BBS soil ($H'=2.24$) was similar to the UOF soil ($H'=2.49$), whereas BOF showed the lowest diversity ($H'=1.23$; Fig. 3B). Pielou's evenness index also was equal for UOF and BBS soils ($J'=0.87$ for both sites) and lowest ($J'=0.56$) at the BOF site where the effect of the fire was most intense (Fig. 3B).

Three years after the wildfire, the diazotrophic community at the rhizosphere of burned holm-oaks was dominated by *Azospirillum*, the burned soil with the legume *A. decorticans* was dominated by the genus *Rhizobium*, whereas in the unburned rhizosphere there was a mixture of five nitrogen-fixing genera of the Proteobacteria.

Protein sequence analysis showed that most of the NifH sequences were from phyla Proteobacteria and Firmicutes in all the sites. These two phyla accounted for 100% of the sequences from the BOF site (Fig. 3C) while at UOF and BBS sites around 2% of the sequences belong to other phyla. The main difference among sites was the high proportion of Firmicutes phylum (15.09%) in the BOF soil compared with the 7.84% in the unburned soil and 4.00% in the UOF soil. Detailed NifH sequence comparisons at the genus level showed that at the BBS site, where a leguminous shrub was predominant, the most abundant genera were *Rhizobium*, *Methylobacterium* and

Bradyrhizobium (Table 2); these genera of the Proteobacteria are well-known nitrogen-fixers in symbiosis with legume plants. The abundance of *Rhizobium* was significantly higher ($p = 0.01$) at the BBS site, compared to BOF and UOF sites where its abundance was very low, whereas the genus *Bradyrhizobium* was present in the UOF and BBS, but absent in the BOF site. In the soils from the BOF site, the characteristic genera were *Azospirillum* (Proteobacteria) and *Paenibacillus* of the Firmicutes, but only the first was significantly more abundant compared to the other sites. The unburned UOF site had the highest diversity but there was no clear predominance of one genus, with a roughly equal presence of *Methylobacterium*, *Azospirillum*, *Bradyrhizobium*, *Rhodopseudomonas* and *Geobacter*.

Discussion

Although the ratios of carbon to nitrogen (C/N) of the soils were similar at 5 – 25 cm depth, the effect of the wildfire on the soil was observed in the reduced concentrations of total nitrogen (N) and C in the soils (Table 1) from the burned sites (BOF and BBS). The similar C/N ratios of burned and unburned soils contrasts with other studies reporting an increase [33, 34] or decrease [35, 36] in the C/N ratio of the soil after burning. Nevertheless, since our analysis was made three years after the fire, this similar C/N ratio could be an indication of ecosystem recovery. Moreover, our results are consistent with those of previous studies reporting decreases in organic matter content and total N in burned soils in the years following the fire [8, 9, 37]. However, the effect of the fire on the other analysed parameters is not so clear, since pH in BBS (non-rhizospheric burned bulk soil) is similar to that of UOF, but with an alkalisation at the BOF site (Table 1). This increase of pH may be due to the presence of burned holm oak trees, according to other authors [16, 37]. Therefore, three years after the wildfire the

main soil factors that influenced the holm-oak rhizospheric communities are pH and the decreased content of C and N.

The metagenomic analyses, as an indicator of the potential pathways of the nitrogen cycle, showed no differences between the burned and the unburned rhizospheres at the level of metabolic groups, including that of nitrogen metabolism. However, the detailed examination of this function showed that at the subsystem level 3 (functions of the nitrogen cycle) there were some statistically significant differences among sites. Thus, in the burned rhizosphere, with 0.233% total N concentration, there were relative decreases of the dissimilatory nitrite reductase and the denitrification functions and an increase of allantoin utilization (Fig. 1), compared to the unburned rhizosphere, with 0.366% total N. The main functions identified in both rhizospheres were ammonia assimilation and the ammonification of nitrate and nitrite, which, together with cyanate hydrolysis, allantoin utilization and N₂-fixation, reflect the pathways of N incorporation to the ecosystem by the bacterial communities. However, the potential N cycle in the BOF rhizosphere can be seen as conservative because the percentage of sequences related to allantoin utilization (N influx to the metabolic pathways) was higher than in UOF, moreover the percentage of sequences for denitrification and dissimilatory nitrite reductase functions (N efflux) was lower in BOF than in UOF (Fig. 1). Our data shows that at the BOF site, the N-metabolism of the rhizospheric community is likely to limit losses of nitrogen with an increased potential pathway of N-incorporation from plant origin, such as allantoin. The most obvious functions involved in N losses from the rhizosphere of unburned holm oak are the higher levels of denitrification pathway and the dissimilatory nitrite reductase activity, together with the nitrosative stress and nitric oxide synthase. Nitric oxide (NO) is involved in all of these pathways of N losses, since it is formed as an intermediate step during denitrification, but also as a signalling and

defence molecule of major importance [38]. These functions show that large losses of N are likely to occur via gaseous emissions. Goberna et al. [39] concluded that immediately after a prescribed fire, the biogeochemical cycling in Mediterranean shrublands becomes less conservative, due to an increase in microbial biomass and activity, and changes in the structure of the bacterial community. In our study, the analysis was performed three years after the wildfire, in a context of possible N loss by volatilization and leaching. Thus, the microbial communities of burned rhizospheres tend to incorporate N from alternative N sources, as shown by the higher abundance of sequences related to allantoin utilization and nitrogen fixation. It is important to note that allantoin in soil is derived from eukaryotes, possibly from the burned wood, which would stimulate bacteria *versus* fungi, and Gram-positive *versus* Gram-negative bacteria [40]. In general, the number of sequences of the different N functions showed a conservative ecosystem for the nitrogen cycle in burned and unburned soils. However, the unburned rhizosphere is a wild, developed system, with a consistent resource use and therefore may display more nitrogen loss through denitrification and dissimilatory nitrite reductase activity.

In spite of the deep sequencing effort, no sequences of the nitrogenase reductase gene (*nifH*) were obtained. Since this is the marker gene for the N-fixation pathway, we employed *nifH* clone libraries to study the fire effect on this pathway. Burning has frequently been reported to have a detrimental effect on N-fixing bacteria in the soil, decreasing their diversity, richness and biomass [12, 16, 41]. The presence of Proteobacteria and Firmicutes in the NifH libraries (Fig. 3C) are consistent with the results of Yeager et al. [12], who found NifH sequences from these phyla plus Cyanobacteria, on soils of a ponderosa pine forest between one and 14 months after a fire. Moreover, our results indicate that the species richness and the diversity of NifH

sequences were lower in burned (BOF and BBS) than in UOF soils (Fig. 3), with the lowest values obtained at the site most severely affected by the fire (BOF). The results of Kennedy and Egger [16] suggested that the presence of living trees during the fire, influences the soil microbial communities more than an intact forest floor, and this observation was corroborated by Switzer et al. [37] who demonstrated that soils from burned living trees have smaller bacterial populations than cut trees or stacked wood. Thus, after the fire, the rhizosphere of holm-oak (BOF) was more affected than soils with herbaceous vegetation (BBS), as shown by the diversity indices, the number of OTUs and the rarefaction curves (Fig. 3, Fig. S2). On the other hand, the very similar values of diversity between UOF and BBS sites could be due to the proliferation of a nitrogen-fixing shrub in BBS after the fire.

The phyla Bacteroidetes and Actinobacteria were two of the most abundant at all sites, according to the similarity of the proteins of the N-cycle in the metagenomic analysis (Fig. 2), but only one sequence of each phylum was obtained in the analysis of the NifH diversity. This discrepancy could reflect the lower proportion of diazotrophic microorganisms of these taxa in compared to the Proteobacteria [42]; only the phylum Firmicutes shows similar proportional abundances using both approaches (gene libraries and metagenomics) in the BOF rhizosphere. The increase of both Gram-positive phyla (Firmicutes and Actinobacteria) after the wildfire is in agreement with the results of other authors on wildfires [10, 12] and the presence of functional genes associated with allantoin utilization in soil [40]. The observed differences in the structure and the composition of the Actinobacteria communities probably reflect the ability of many of the bacteria of this group to form spores and proliferate after adverse events *via* sporulation; at the same time, specific genera like *Arthrobacter* are adapted to oligotrophic conditions. Gram-positive bacteria can withstand high temperatures and

proliferate on partially sterile burned soils in the form of spores [10, 12, 43]. These results are consistent with those of other studies reporting an increase in the relative abundance and richness of bacteria within the phylum Actinobacteria in biochar-treated soils after six months of incubation [44], or an increase in actinobacterial colony-forming units on soils 32 months after a fire [10]. Within the phylum Actinobacteria, the largest differences among sites in the phylogeny related to the N cycle was due to increased abundance of the genus *Arthrobacter* in the BOF rhizosphere (Fig. 2B). This genus has been related to the degradation of aromatic compounds [45], which appear in the soil after a wildfire, therefore it could play a key role in the recovery of the microbial community facilitating the pass from oligotrophic to a copiotrophic conditions. Similarly, the presence of the genus *Azospirillum* as a diazotrophic microorganism in the BOF rhizosphere (Table 2) could reflect its importance at the onset of ecosystem recovery, since it has been described as a plant growth-promoting rhizobacterium increasing root development [46]. The increase of the *Arthrobacter* genus after wildfire is important, as it was the second-most abundant in the BOF rhizosphere, and the genus with strongest significant differences between unburned and burned holm oak rhizospheres (Fig. 2B).

In combination, these results show a modification of microbial community structure, with an increase of the Gram-positive phyla, as a consequence of a wildfire. These bacteria fulfil a range of potential functions in the N-cycle, with a major role for the phylum Firmicutes in nitrogen-fixation and for the phylum Actinobacteria in other potential pathways of the nitrogen cycle associated with the holm oak rhizosphere. After a wildfire, these pathways showed a greater tendency towards the conservation of nitrogen in the ecosystem, with an increase in functions associated with N retention (allantoin utilization) and a decrease in functions associated with N losses

(denitrification and dissimilatory nitrite reductase) in the burned rhizosphere. Due to its high abundance and potential important role in N-cycling, the phylum Actinobacteria merits further attention as a biomarker for ecosystem recovery after wildfire.

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Table 1. Soil chemical and physical properties at 5 – 25 cm depth within sampled areas of unburned holm-oak forest (UOF), burned holm-oak forest (BOF) and burned bulk shrubland soil (BBS) three years after wildfire in Sierra Nevada National Park, South Eastern Spain.

Parameter	UOF	BOF	BBS
Clay (%)	21.00	20.50	12.05
Sand (%)	45.74	49.54	56.08
Silt (%)	33.26	29.96	31.87
Type of soil	Loam	Loam	Sandy loam
pH (H ₂ O)	6.1	7.6	6.5
pH (KCl)	5.7	7.0	6.0
Available water (%)	17.11	16.43	15.20
Salinity (mS/cm)	0.14	0.22	0.08
Organic matter (%)	7.61	4.54	4.54
Total N (%)	0.366	0.233	0.250
C/N ratio	11.95	11.19	10.44
Assimilable Phosphorous (mg/kg)	8	5.2	20
Magnesium (mg/kg)	4.36	7.20	1.48
Calcium (%)	12.65	17.97	8.65

Table 2. Phylogenetic assignment of *nifH* gene sequences at phylum and genus levels. The numbers indicates the numbers of sequences detected within sampled areas of burned bulk soil (BBS), burned holm-oak forest (BOF) and unburned holm-oak forest (UOF) three years after wildfire in Sierra Nevada National Park, South Eastern Spain.

		BBS	BOF	UOF	Total
Proteobacteria	<i>Gluconacetobacter</i>	2	0	0	2
	<i>Azospirillum</i>	1	33	9	43
	<i>Bradyrhizobium</i>	6	0	7	13
	<i>Rhodopseudomonas</i>	2	1	7	10
	<i>Rhizobium</i>	22	2	0	24
	<i>Ensifer</i>	2	1	0	3
	<i>Methylobacterium</i>	7	0	12	19
	<i>Zymomonas</i>	0	0	1	1
	<i>Azorhizobium</i>	0	3	1	4
	<i>Skermanella</i>	3	2	2	7
	<i>Methylocystis</i>	0	1	0	1
	<i>Methylovirgula</i>	0	1	0	1
	<i>Rhodocista</i>	1	0	0	1
	<i>Cupriavidus</i>	0	1	1	2
	<i>Geobacter</i>	0	0	7	7
Firmicutes	<i>Paenibacillus</i>	4	7	1	12
	<i>Clostridium</i>	0	0	1	1
	<i>Pelosinus</i>	0	1	0	1
Actinobacteria	<i>Arthrobacter</i>	0	0	1	1
Bacteroidetes/Chlorobi	<i>Dysgonomonas</i>	1	0	0	1
		51	53	50	154

Figure captions

Fig. 1. Percentage of nitrogen metabolism sequences, by function, according to the subsystem classification of the MG-RAST web-server. Black bars correspond to the metagenome of the burned holm-oak rhizosphere (BOF) and grey bars to the metagenome of undisturbed (UOF) rhizosphere. Numbers beside bars indicate the percentage of proteins corresponding to the function in each sample when significantly ($p < 0.05$) different between burned and non-burned rhizospheres.

Fig. 2. Bacterial taxa involved in the metabolism of the nitrogen cycle (metagenomic sequences associated to ko00910, nitrogen metabolism, of KEGG orthology database). a) Percentage of the bacterial phyla in the burned (BOF, black bars) and in the undisturbed (UOF, grey bars) rhizospheres. b) Relative abundance of those bacterial genera with a proportion higher than 2% of the nitrogen metabolism sequences of its respective metagenome. Asterisks represent a statistically significant ($p < 0.05$) difference between burned and undisturbed rhizospheres.

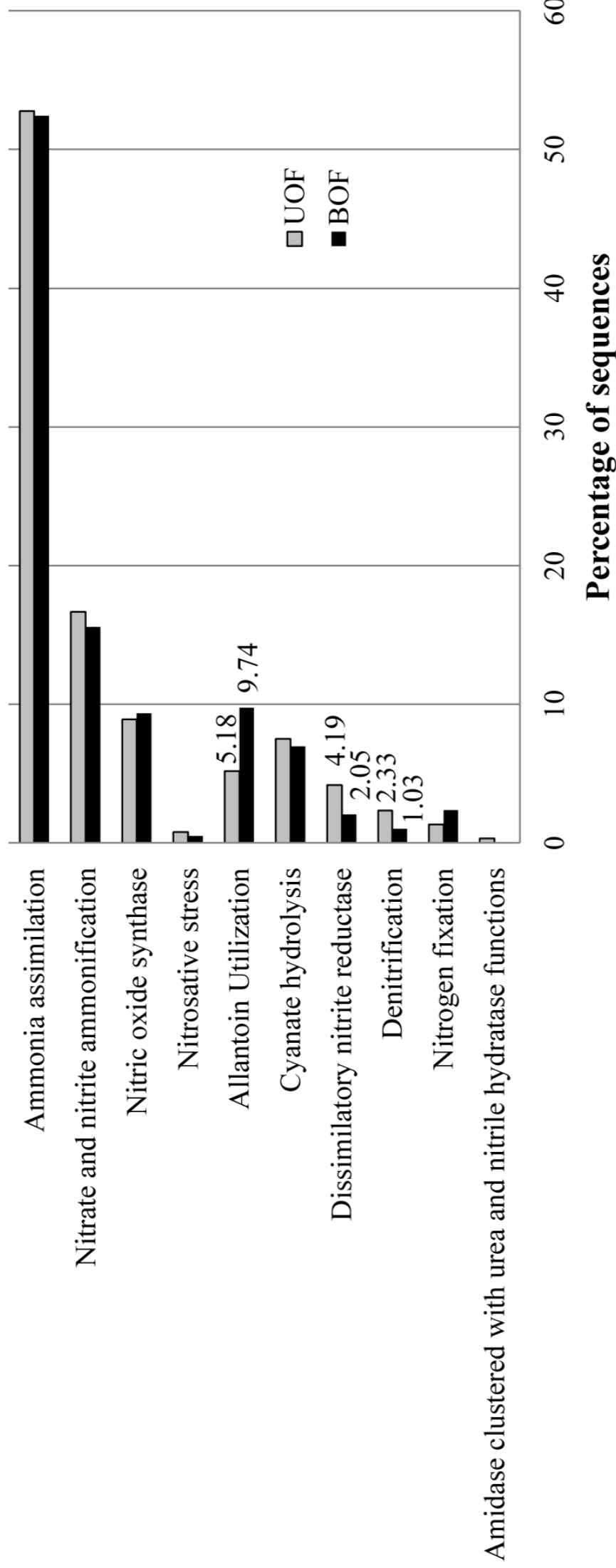
Fig. 3. Ecological indices and proportion of bacterial phyla for the *nifH* clone libraries. The names below the bars correspond to the sampled rhizospheres UOF (undisturbed holm-oak forest), BOF (rhizosphere of burned holm-oak forest), BBS (burned bulk soil). a) Observed OTUs and Chao1 index of estimated richness. b) Shannon-Wiener index of diversity and Pielou index of evenness. c) Relative abundance of NifH sequences from phyla detected in the sampled sites. The phyla considered are Proteobacteria (■), Firmicutes (□), Bacteroidetes (▒) and Actinobacteria (■). OTUs, operational taxonomic units defined by a 93% DNA similarity cut-off for the *nifH* gene.

Supplementary Material

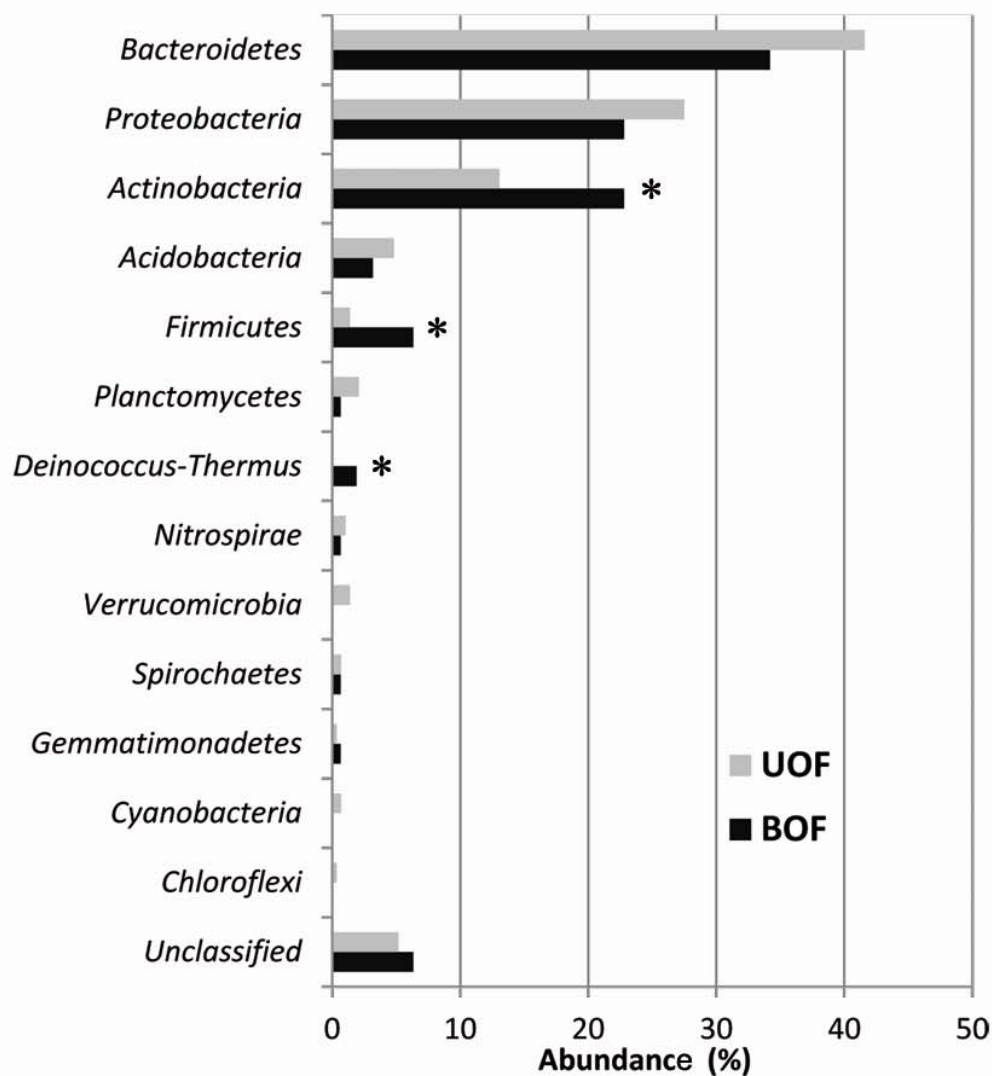
Fig. S1. Description of the sampled areas. A) Location of study areas with its GPS position coordinates, altitude and kind of vegetation. B) Geographic location of the sampled sites at Sierra Nevada. UOF, undisturbed holm-oak forest; BOF, burned holm-oak forest; BBS, burned bulk soil with grasses and shrub before the wildfire.

Fig. S2. Rarefaction curves (A) and coverage table (B) for the *nifH* gene clone libraries. UOF, undisturbed holm-oak forest; BOF, burned holm-oak forest; BBS, burned bulk soil with grasses and shrub before the wildfire.

Fig. S3. Agglomerative hierarchical clustering of the NifH proteins of each library with the Euclidean distance matrix and UPGMA algorithm. Analyses were carried out on the abundance of each OTU defined by a 93% similarity cut-off. The name of each branch corresponds to the sampled site: UOF (undisturbed holm-oak forest), BOF (rhizosphere of burned holm-oak forest), BBS (burned bulk soil).

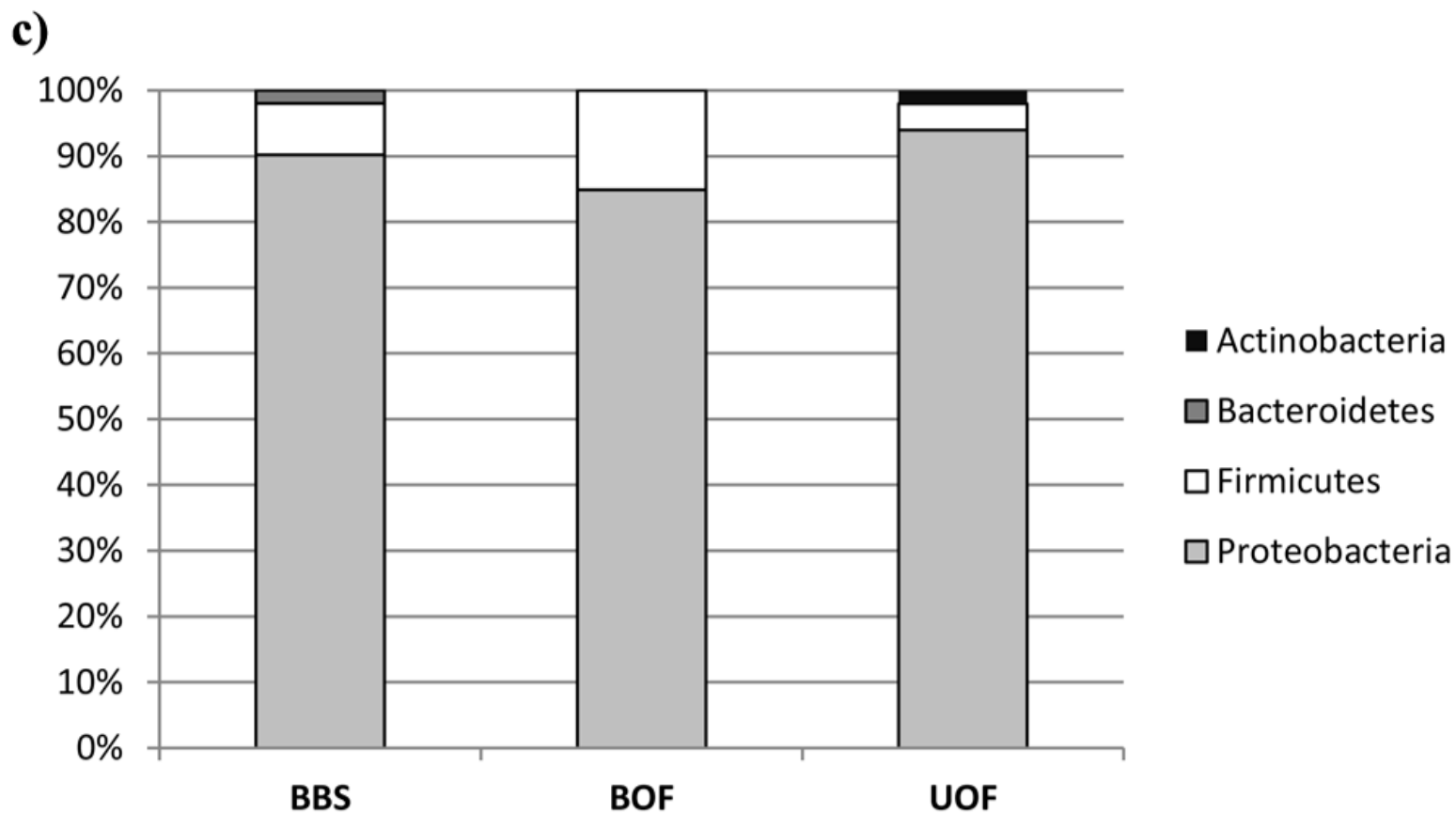
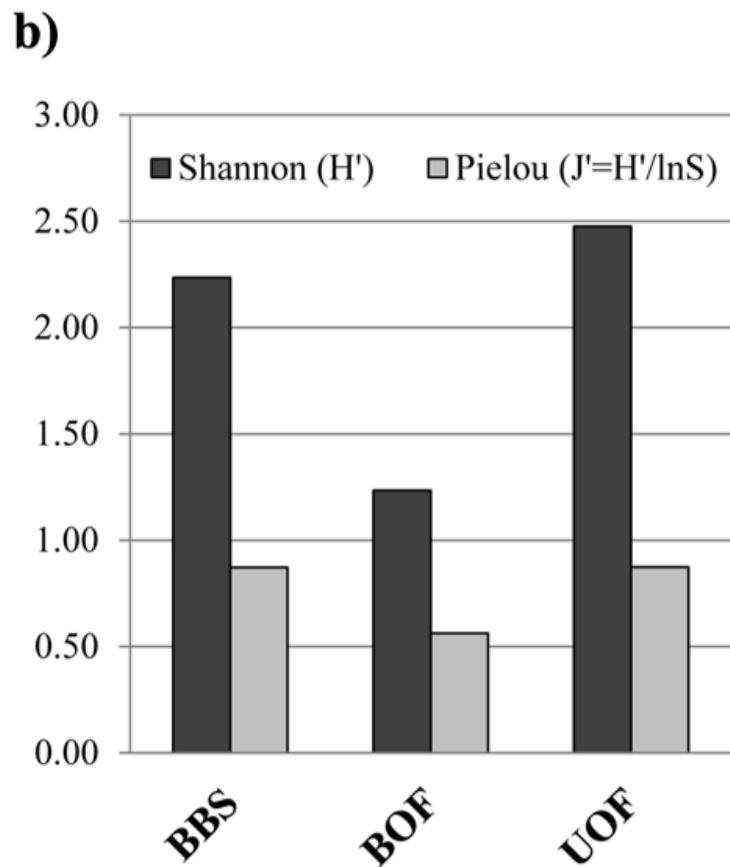
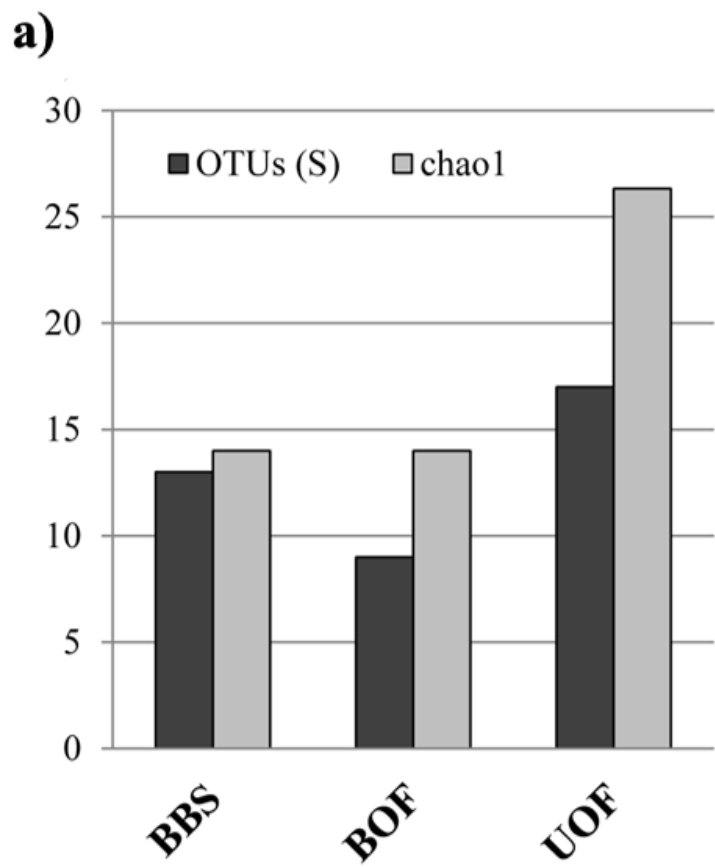


A)



B)

Phylum	Genus	UOF	BOF
<i>Bacteroidetes</i>	<i>Niastella</i>	11.34	10.13
<i>Proteobacteria</i>	<i>Bradyrhizobium</i> *	7.22	1.27
<i>Bacteroidetes</i>	<i>Flavobacterium</i>	5.84	2.53
<i>Actinobacteria</i>	<i>Arthrobacter</i> *	0.00	6.96
<i>Bacteroidetes</i>	<i>Chitinophaga</i>	1.72	4.43
<i>Bacteroidetes</i>	<i>Mucilaginibacter</i>	2.75	2.53
<i>Actinobacteria</i>	<i>Mycobacterium</i>	1.37	3.16
<i>Bacteroidetes</i>	<i>Pedobacter</i> *	3.44	0.63
<i>Bacteroidetes</i>	<i>Solitalea</i>	2.75	1.27
<i>Proteobacteria</i>	<i>Burkholderia</i>	2.41	1.27
<i>Bacteroidetes</i>	<i>Niabella</i>	2.41	1.27
<i>Firmicutes</i>	<i>Bacillus</i> *	0.34	3.16
<i>Actinobacteria</i>	<i>Streptomyces</i>	2.06	1.27
<i>Actinobacteria</i>	<i>Blastococcus</i> *	0.00	2.53
<i>Bacteroidetes</i>	<i>Spirosoma</i> *	0.00	2.53



A)

Site	Altitude	Orientation	Type of are	Location
UOF	1790 masl	South	Holm-oak forest unburned	N 36°58'11", W 3°27'37"
BOF	1566 masl	South	Holm-oak forest burned, resprout of Holm-oak trees	N 36°57'26", W 3°27'48"
BBS	1610 masl	South	Shrubs and grass burned	N 36°57'40", W 3°27'42"

B)

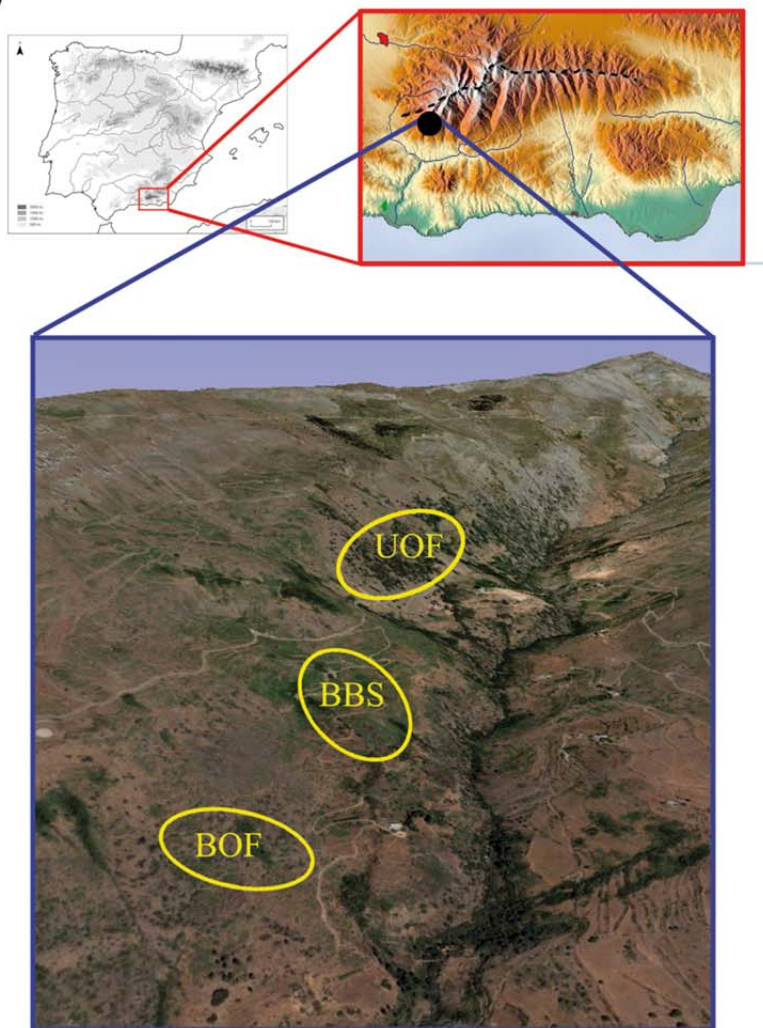


Fig. S1. Description of the sampled areas. A) Location of study areas with its GPS position coordinates, altitude and kind of vegetation. B) Geographic location of the sampled zones at Sierra Nevada. UOF, undisturbed holm-oak forest; BOF, burned holm-oak forest; BBS, burned bulk soil with grasses and shrub before the wildfire.

Determination of soil parameters.

Texture of soil samples was measured by Bouyoucos hydrometer method (Bouyoucos, 1962). Available water measurement was carried out by gravimetry after drying at a maximum temperature of 105 °C (Gardner, 1986). Potentiometric method (Willard et al., 1974; Bates, 1983) is used for the determination of pH. The method of electrical conductivity was used for determine the salinity. Quantification of total organic carbon was made by volumetric techniques with wet oxidation at controlled temperature (Mebius, 1960). Soluble P was measured by Bray method (Bray and Kurtz, 1945), and quantification is performed by colorimetry. Total nitrogen determination was performed with the Kjeldahl method (Bremner, 1965). Determination exchangeable bases (Ca_2^+ and Mg_2^+) soil was made by spectrometry using ammonium acetate.

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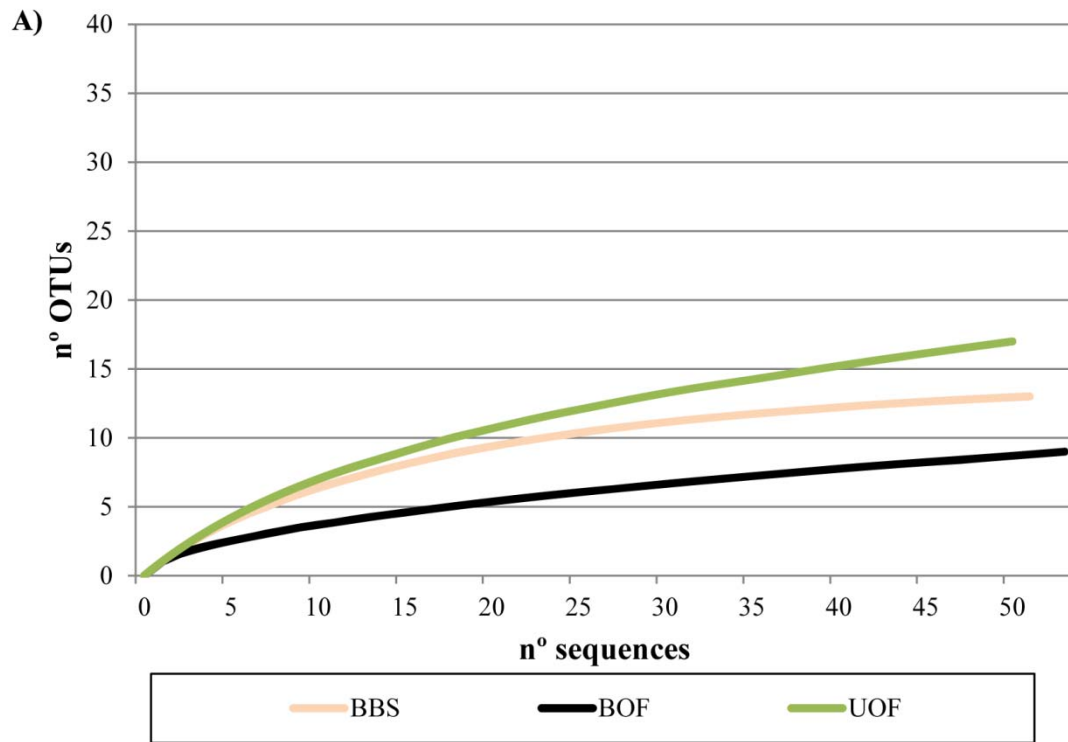
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B)

Zone	BBS	BOF	UOF
Coverage (%)	94.12	90.57	84.00

Fig. S2. Rarefaction curves (A) and coverage table (B) for the *nifH* gene clone libraries. The sampled zones were: UOF, undisturbed holm-oak forest; BOF, burned holm-oak forest; and BBS, burned bulk soil with grasses and shrub before the wildfire.

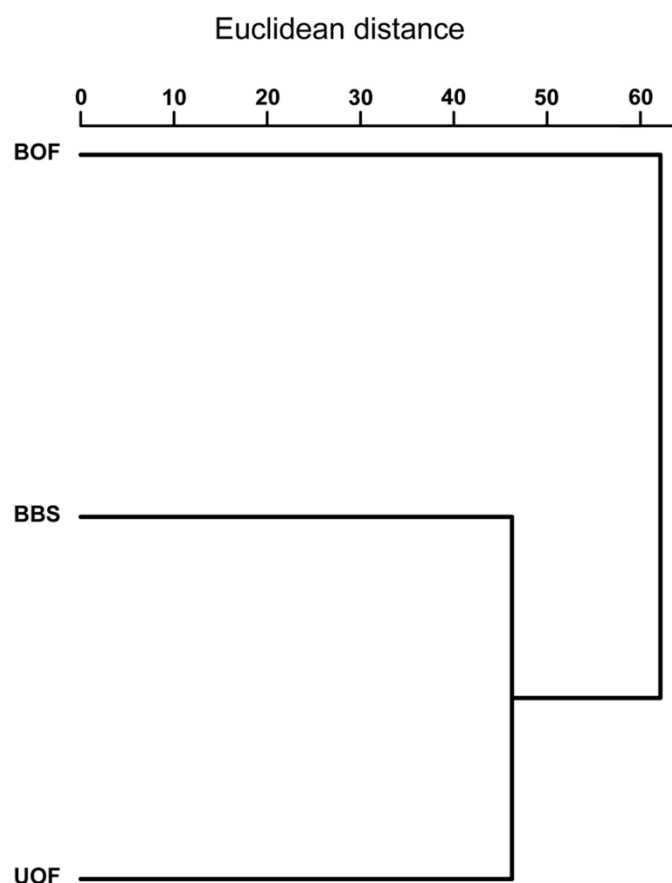


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